Environmental Health Perspectives
Vol. 68, pp. 11-17, 1986

Association of Carcinoma Yield with Early Papilloma Development in SENCAR Mice

by Richard J. Bull,* Merrel Robinson,† and R. Dana Laurie†

The responsiveness of SENCAR mouse skin to 20 different chemicals with known carcinogenic properties was assessed in initiation/promotion experiments. The purpose of these experiments was to evaluate the extent of false negative responses in mouse skin initiation/promotion protocols and to determine the extent to which early papilloma development can be used to predict the eventual development of malignant tumors. The chemicals were administered as initiators by four different routes: oral, intraperitoneal, subcutaneous, and topical. Following the initiating dose of carcinogen, the animals were subjected to topical applications of 1 μg 12-O-tetradecanoylphorbol-13-acetate (TPA) 3 times per week for a period of 20 weeks. The yield of papillomas at 24 weeks was selected as a potential predictor of carcinoma yields at 52 weeks following the start of the promotion schedule. Positive responses were observed with only eight of the compounds tested. Where positive results were observed, there was some evidence that the response could depend both qualitatively and quantitatively on the route of administration. However, no route was clearly superior, i.e., different chemicals gave greater responses by different routes. Papilloma yield at 24 weeks following the start of the promotion schedule was clearly related to the development of carcinomas at 52 weeks. No simple linear relationship existed between papilloma yield and carcinoma development, since the number of malignant tumors per papilloma decreased with increasing papilloma yields. The relationship between papilloma and carcinoma yields appeared to be independent of the carcinogen used. These data indicate that there are some limitations in using mouse skin initiation/promotion experiments as the sole basis for identifying substances with carcinogenic activity. However, the test does perform well within certain classes of compounds. Within the limits of these chemical classes, the use of papilloma yield to predict carcinoma yield appears justified.

Introduction

There are a variety of reasons for pursuing animal models that display a high degree of responsiveness to particular stimuli. From the point of view of fundamental research, a strain or stock of animals that is particularly susceptible to a given disease state provides a tool for identifying some of the key steps involved in the development of the disease and thus provides insights into the treatment and/or prevention of the disease. From the context of an agency that is responsible for ensuring that dangerous chemicals are not introduced into the environment, a sensitive strain provides the possibility of simplifying testing schemes.

Our interest in the SENCAR stock was two-fold: (1) could the increased sensitivity of the skin in this stock be used to broaden the classes of chemical carcinogens that could be detected and (2) could papilloma development be used as an accurate predictor of the ultimate incidence of malignant tumors? If these two questions could be answered in the affirmative, it would allow a much broader use of the skin tumorigenesis model and would confirm its inherent convenience as a short-term test method in environmental carcinogenesis. Particularly, we hoped that we could take advantage of the rapid development of papillomas in these mice resulting from the initiation/promotion protocols employing 12-O-tetradecanoylphorbol-13-acetate (TPA) as a tumor promoter. The short time involved in the development of these tumors and the fact that they are an easily identifiable external lesion would make SENCAR mice an ideal system for routine testing of tumor-initiating agents.

To establish the extent to which SENCAR mice might fit this ideal, we have subjected 20 different carcinogens, representing 14 different chemical classes, to tests as tumor initiators (*1*). We also chose to explore the extent to which the route of administration influences the response to these chemicals in a classical initiation/promotion protocol.

Materials and Methods

Chemicals

All compounds tested as initiators were purchased at the highest purity available and submitted for analysis by a contract laboratory, Environmental Health Research and Testing, Inc. (Contract No.68-03-1720, M.

*College of Pharmacy, Washington State University, Pullman, WA 99164-6510.

†Toxicology and Microbiology Division, U.S. Environment Protection Agency, Cincinnati, OH 45268.

Pereira, project officer). The results of these analyses and the source of each chemical is provided in Table 1. Only in the case of 2-acetylaminofluorene, which was found to be only 50% pure, does it appear that the level of purity was so low as to make the results reported here somewhat questionable. Since the experiment had essentially been completed before the purity analyses were reported, the results of tests using 2-acetylaminofluorene are included in this report. TPA was obtained from Chemical Carcinogenesis (Eden Praire, MN).

Animals

Female SENCAR mice were used in all experiments. As indicated in Table 1, mice were obtained either from Oak Ridge National Laboratories (Oak Ridge, TN), or from Harlan Sprague-Dawley (Indianapolis, IN). Animals were begun on study when 6 to 8 weeks of age and were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO) and distilled water provided *ad libitum*.

Experimental Design

Depending on the intent of the experiment, each group of animals contained either 40 (first round screening), 30, or 25 mice (when multiple doses were used to confirm positive results and to establish dose-response relationships). Table 2 presents the doses of each chemical that produced the largest response by each route of exposure. The backs of the animals were shaved 3 days before application of the test chemical (tested as initiators, only) and once weekly during the tumor promotion period. Using available acute toxicity data, the initial doses to be used in the screening phase were selected to approximate one-fifth the LD_{50}. Additional doses were tested if a positive response was indicated. On occasion, if the initial dose resulted in a marginal response, higher total doses were administered by splitting the initiating dose into six equal doses that were applied over a 2-week period. Depending on the compound, either acetone or ethanol was used for topical applications and emulphor, saline or water used as the vehicle for systemic routes of administration. Control groups received equivalent amounts of the appropriate vehicle.

Two weeks following the administration of the last dose of the test chemical, a promotion schedule was begun, involving the application of 1.0 μg TPA in 0.2 mL acetone to the back of each animal three times weekly. The topical route was always used for TPA administration regardless of the route by which the test chemical was administered. At the highest dose of each test chemical used, a group receiving acetone applications in place of TPA applications was included. These groups were usually limited to 20 animals and served to determine whether a single dose of the chemical could induce tumors in the absence of TPA promotion. In no case were significant numbers of tumors observed in

Table 1. Source and analysis of carcinogen test matrix chemicals and source of SENCAR mice used to test each compound.

Compound	Source	Lot #	% Purity	Contaminants		SENCAR source[a]
2-Acetylaminofluorene	Sigma, St. Louis, MO	1092–0202	50			HSD
				Carbonic acid	30.0%	
				2-Methoxybenzoic acid methyl ester	14.0%	
				Triphenylmethane	4.1%	
Aflatoxins	Sigma	117C-0218	CND			OR
Azaserine	Sigma	72F-0401	99+			HSD
Azobenzene	Eastman, Rochester, NY	B7A	99+			OR
Benzo(a)pyrene	Sigma	15C-0116	99+			OR
1,2-Dibromoethane	Fluka, Hauppague, NY	171756104	92	Bromochloroethane	5.0%	HSD
				Dibromopropane	0.3%	
				Tribromomethane	0.3%	
				Tribromomethane	1.7%	
Diethylnitrosoamine	Sigma	49C-0348	99+			OR
				1,2,3-Trichloropropene	0.2%	
1,2-Dimethylhydrazine	Aldrich, Milwaukee, WI	092497	98			HSD
1,4-Dioxane	Aldrich	101197	99+			HSD
Ethyl carbamate (urethane)	Aldrich	JDO31997	99+			OR
Lead acetate	Fisher, Cincinnati, OH	726062	99+			OR
3-Methylcholanthrene	Fluka	66230	99+			HSD
Methymethanesulfonate	Aldrich	4120TD	99			OR
N-[4-(5-nitro-2-furyl)-2-thiazoyl]-formamide (FANFT)	Saber Lab, Morton Grove, IL	791101	99+			OR
2-Napthylamine	Sigma	18C-0440-1	99			HSD
S-(2-propenyl)-1,3-benzodiazole (Safrole)	Aldrich	050197	90.2	Camphor	0.8%	HSD
				Cadinenes scans	4.9%	
β-Propiolactone	Sigma	109C-0516	96	2-Oxopropanoic acid	1.5%	OR/HSD
				Acetic acid scans	2.4%	
2,4,6-Trichlorophenol	Eastman	B7B	99+			OR

[a] CND = could not determine; OR = Oak Ridge National Laboratory; HSD = Harlan Sprague-Dawley.

Table 2. Summary of positive and negative results with chemical carcinogens tested as tumor initiators in the skin of female SENCAR mice by different routes of administration.

Compound	Dose producing largest response, mg/kg	Route of exposure[a]			
		Oral	IP	SC	Topical
2-Acetylaminofluorene	200	--	NT	--	--
Aflatoxin B_1	1.5	--	NT	--	--
Azobenzene	200	--	NT	--	--
Azaserine	30	--	NT	+ +	NT
	200	NT	NT	NT	--
Benzene	800	--	NT	--	--
Benzo(a)pyrene	50	NT	NT	NT	+ +
	100	NT	+ +	+ +	NT
	500	+ +	NT	NT	NT
Dibromoethane	50	--	NT	--	NT
	200	NT	NT	NT	--
Dibutylnitrosamine	200	--	NT	--	NT
	400	NT	NT	NT	--
Diethylnitrosamine	50	--	NT	--	--
Dimethylbenzanthracene	0.06	NT	NT	NT	+ +
	5	+ +	NT	NT	NT
	1.3	NT	+ +	+ +	NT
1,4-Dioxane	1000	--	NT	--	--
Ethyl carbamate	300	NT	+ +	NT	NT
	500	+ +	NT	+ +	+ +
FANFT	500	--	NT	NT	--
	1000	NT	NT	+ +	NT
Lead acetate	600	--	NT	NT	--
3-Methylcholanthrene	250	+ +	NT	+ +	NT
	500	NT	NT	NT	+ +
Methylmethanesulfonate	40	--	NT	--	--
Methylnitrosourea	30	+ +	+ +	+ +	+ +
β-Propiolactone	100	--	NT	--	+-
	800	--	NT	NT	+ +
Safrole	470	--	NT	--	--
2,4,6-Trichlorophenol	200	--	--	--	--

[a] NT = not tested, -- = negative, + + = positive, +- = equivocal.

these groups. Consequently, the results were omitted from this paper to simplify presentation.

Tumor incidences were recorded weekly for the first 24 weeks following the start of promotion, and monthly thereafter. To be included in the cumulative count, a tumor had to be at least 1 mm in diameter and be present in the same location for three consecutive weeks. At 52 weeks, the experiments were terminated and histological evaluations performed for all gross lesions that were noted at necropsy. Some experiments were terminated before 52 weeks when they were clearly negative (< 0.1 tumor/animal) at all doses tested at 24 weeks.

Results

Table 2 summarizes the data obtained from tests of all chemicals in terms of positive or negative responses. Chemicals were judged positive if the response was significantly different from the concurrent control group (using a multiple cell contingency test based on the number of animals bearing tumors, at $p < 0.01$) and if a response of greater than 0.4 tumors/animal was observed in a group of 40 animals. A positive response was also indicated if the tumor yield was between 0.3 and 0.4 tumors/animal, if the results were confirmed in a separate experiment involving multiple doses, and if the results increased the statistical confidence relative to the first experiment. Overall, the control response to TPA promotion in the absence of prior treatment with a chemical was 0.08 tumor/animal (total of 50 tumors in 622 animals). However, this value varied considerably between experiments and indicates the need for caution in accepting the statistical evaluation of a single experiment when a low-level response is observed.

The results in Table 2 indicate that eight of the 20 chemicals tested for their ability to initiate skin tumors in SENCAR mice were found to be positive by one or more routes of administration. Six of the eight positive compounds were found to initiate tumors when applied topically, the usual route of administration. Seven of the eight compounds were positive by the subcutaneous route (administered in the back), and five of the eight were positive if the chemical was administered orally.

When administered orally, the three polycyclic aromatic hydrocarbons, i.e., (PAHs) [7,12-dimethylbenz(a)anthracene, (DMBA), benzo(a)pyrene [B(a)P], and 3-methylcholanthrene (MCA), ethyl carbamate, and methylnitrosourea were positive. The topical route of exposure added α-propiolactone to the list of positive compounds. This was the only route by which β-propiolactone produced a significant response. *N*-[4-(5-Nitro-2-furyl)thiazolyl]formamide (FANFT) and azaserine were positive only if administered subcutaneously. Testing by the intraperitoneal route was limited to a few compounds, and there were no uniquely positive or negative compounds identified by this route.

Figure 1 provides the dose-response information obtained for the eight positive compounds. The route-specific nature of the responses was not entirely qualitative, since rather marked differences in potency were observed using different routes of administration of the same chemical. PAHs, in particular, are very much more potent when they are topically applied relative to systemic routes of administration (Figs. 1*e*, 1*f*, and 1*g*). In general, the activity observed with PAHs by this route was at least two orders of magnitude greater than the alternate routes. On the other hand, ethyl carbamate was approximately two times as potent in initiating skin tumors by systemic routes of administration as by topical application. The basis for these differences was not clear but may be related to the way in which the activated form of the chemical reached its critical site of action.

In general, responses were observed to increase with dose of the initiating agent. This observation was particularly apparent with the PAHs, in which the multiplicity of tumors continued to increase to 10 or more tumors/animal as the dose was increased. At that point, a decreased response was noted with DMBA. This decrease may be more apparent than real because of the difficulty of accurately scoring tumors as they exceed

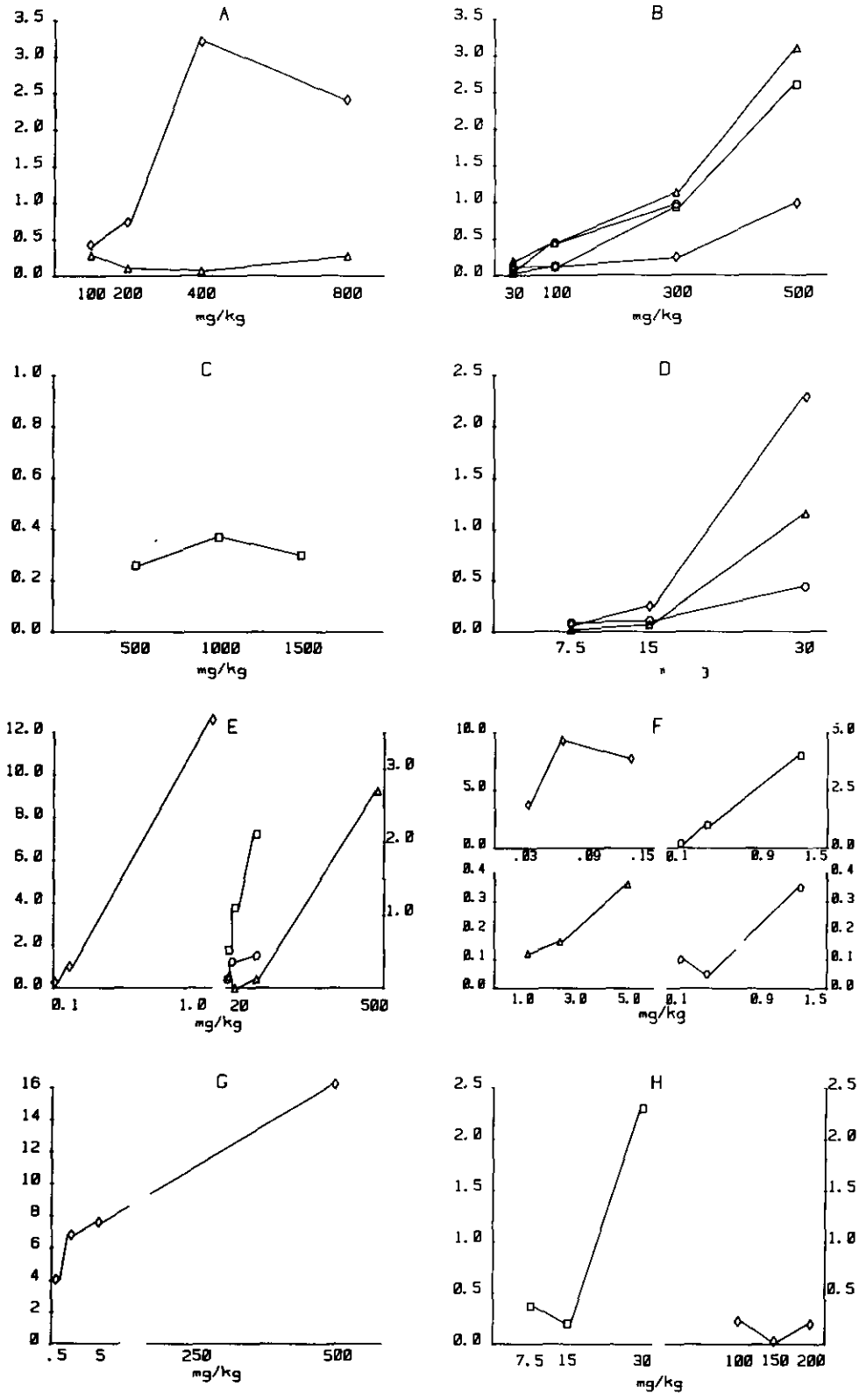

FIGURE 1. Dose-response relationships of chemicals that were found to be positive as tumor initiators in the skin of female SENCAR mice. Data plotted is given as tumors per animal and represents the cumulative papilloma yield observed 24 weeks after beginning the tumor promotion schedule with TPA: (*A*) β-propiolactone, (*B*) ethyl carbamate, (*C*) FANFT, (*D*) methylnitrosourea, (*E*) B(a)P, (*F*) dimethylbenzanthracene, (*G*) 3-methylcholanthrene, (*H*) azaserine. Route of administration of chemicals were: oral (△), topical (◇), subcutaneous (□), and intraperitioneal (○).

this number. On the other hand, the response to β-propiolactone leveled off and even decreased slightly as the tumor yield began to exceed three tumors/animal. This finding apparently reflected a real maximum in the response to β-propiolactone, since an increase in mortality was not observed at the highest dose tested. Similar types of maxima have been observed with other direct-acting initiators (*2*). The limitation on the response to FANFT appears to be much more severe than that observed with the other agents. Responses of similar magnitude were seen with subcutaneous administration of FANFT in other experiments, and the data have repeatedly met our criteria for statistical significance. It is perhaps notable that FANFT was clearly negative by all other routes of administration. Therefore, the possibility that this response was secondary to local irritation produced by FANFT cannot be excluded. Another possible explanation is that the capacity for local metabolism of FANFT is limited: systemic metabolism may have produced metabolites that were not active in the skin. The only other compound that was positive by the subcutaneous route alone, azaserine, produced a much less equivocal result and did not appear to be self-limiting.

The relationship between papilloma development at 24 weeks after the start of the promotion schedule (or 4 weeks following its termination) and the incidence of carcinomas observed in animals maintained on study for a full year is plotted in Figure 2*A*. Twenty-four weeks was chosen for this parameter because statistical evaluations in prior studies indicated that it was optimal for papilloma yields using weakly active compounds. Later times were found to decrease the statistical confidence of the data (*3*). Although the probability of carcinoma development increases as papillomas yield increases, it was clear that there is no simple linear relationship between the development of benign and malignant tumors. Only data obtained from groups that averaged fewer than 14 papillomas/animal were included in the figure to minimize the problem of scoring coalesced tumors. Another reason for limiting the results to a maximum response was that the yield of carcinomas at 52 weeks per papilloma at 24 weeks approximated 0.1. Higher doses resulted in earlier development of fatal malignant tumors. These early deaths obviously distort any simple relationship between the two tumor types at these two time intervals. An additional concern about the relationship between carcinoma development versus early papilloma yield was that the upper portions of the curve (i.e., greater than two papillomas/animal) is dominated by data from PAHs; no other chemical produced an average tumor yield greater than four papillomas/animal. Consequently, the PAH data and the nonPAH data have been segregated in panels *B* and *C* of Figure 2. The curve has also been confined to tumor yields of less than four tumors/animal to allow a better comparison of the relationship at these lower tumor yields. There are no obvious differences in the relationship between papillomas and carcinomas whether they are induced by PAH or non-PAH carcinogens.

In Table 3 the control animals for those experiments that involved topical applications of the chemicals can be compared across individual experiments. In addition, the reproducibility of the responses of female SENCAR mice to identical doses of benzo(a)pyrene administered as the positive control in a variety of other experiments is also presented. All these animals were subjected to the TPA promotion schedule. It is apparent from these

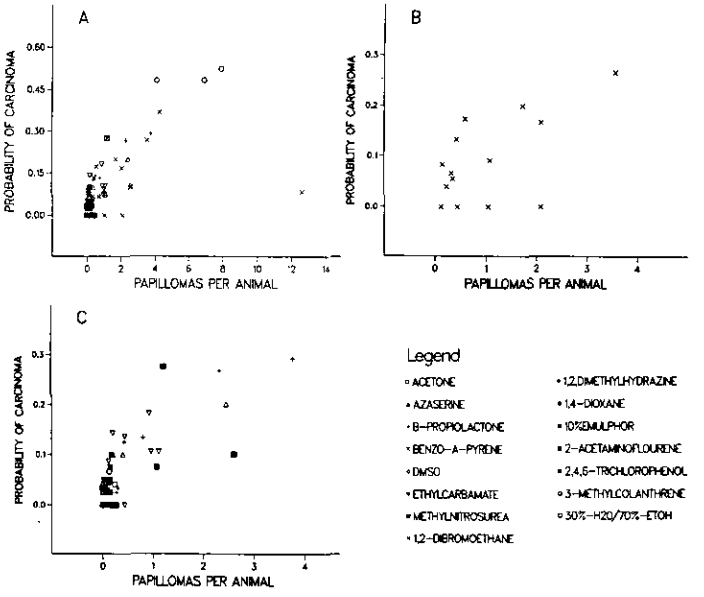

FIGURE 2. Relationship between papilloma yield at 24 weeks following the beginning of the tumor promotion schedule and the carcinoma yield by 52 weeks. (*A*) all the data from experiments that were maintained for the 52-week time period; (*B,C*) expanded scale comparison of this relationship with B(a)P (*B*) and non-PAH initiators (*C*).

Table 3. Reproducibility of tumor yields in control and benzo(a)pyrene-initiated mice following TPA promotion.

Treatment	Trial 1	2	3	4	5	6	7	Sum (Avg)
Benzo(a)pyrene[a]								
N	29	29	15	29	30			132
Papillomas/mouse[b]	3.48	4.44	1.73	0.72	2.10			(2.58)
Carcinomas/mouse[c]	0.27	0.37	0.20	0.07	0.07			(0.22)
Carcinomas/papillomas	0.07	0.09	0.12	0.10	0.08			(0.085)
Acetone								
N	29	40	40	40	40	30	24	243
Papillomas/mouse	0.14	0.08	0.23	0.45	0.03	0.03	0.00	(0.15)

[a] Benzo(a)pyrene was administered topically at a dose of 1 mg/kg body weight. In all cases, 1 μg TPA was applied topically in 0.2 mL acetone 3 times weekly beginning 2 weeks after the initiating dose and continued for 20 additional weeks.

[b] Papillomas/mouse = numbers of papillomas/mouse 24 weeks after beginning promotion.

[c] Carcinomas/mouse = number of carcinomas/mouse 52 weeks after beginning promotion.

data that there is considerable variability in the response of SENCAR mice to a constant dose of B(a)P. However, the relationship between cumulative papilloma yield at 24 weeks and carcinoma development at 1 year remained within fairly narrow limits (i.e., a ratio of 0.07 to 0.12 carcinomas/papilloma) despite a 6-fold variation in the papilloma yield. Similar variation in response was observed in the papilloma yield in animals that received only TPA promotion (except for the application of the vehicle). In this case, the control incidence of papillomas varied from zero to as high as 0.45/animal. Admittedly, the 0.45 value is very rarely encountered (this was only the second time in more than 100 experiments in our experience). However, it does point out the difficulty in accepting marginal responses as significant (e.g., our results with FANFT) without confirmation in separate experiments.

Conclusions

The present study was undertaken as part of a larger effort directed at the question of whether a group of short-term *in vivo* carcinogenesis assays might allow some estimation of carcinogenic hazards without resorting to lifetime feeding studies (*1*). Such an approach had been previously suggested by Weisburger and Williams (*4*). The major reason for choosing multiple tests was that a cursory examination of the literature appeared to indicate that individual test systems based on the responses of a single target organ had relatively high rates of false negative responses. This was definitely the case with the mouse skin initiation-promotion protocols that have used strains of mice less sensitive to tumor initiation than the SENCAR stock (*5*) .

Chemicals used to evaluate the various test systems (*1*) were deliberately chosen to represent a wide variety of chemical classes and to affect a variety of target organs. In some respects, the chemicals selected provided an overly rigorous test of each system's ability to respond. For example, although benzene has been recognized as a human carcinogen for years, it has been very difficult to induce tumors in experimental animals with this chemical. Similarly, the carcinogenic activity of 1,4-dioxane is very low, and questions concerning the mechanism by which lead acetate produces renal tumors pose some difficulties. However, it was felt as a first approximation that the definition of carcinogenic activity must simply refer to the ability to increase tumor incidence in lifetime studies with no regard for potential differences in mechanism.

Despite the above reservations, it was clear that the rate of false negative responses to chemicals with recognized carcinogenic activity is quite high when they are tested as tumor initiators in the mouse skin. There can be no doubt about the carcinogenic activity associated with 2-acetylaminofluorene, aflatoxin B_1, azobenzene, dibromoethane, dibutylnitrosamine, and diethylnitrosamine. Overall, the false negative rate was 60%.

These results do not argue that the mouse skin initiation/promotion assay is without value in evaluating carcinogenic activity of various chemicals. This system appears to be well adapted to the detection of a variety of direct-acting carcinogens (*2*,*3*). It has been routinely used to assay the carcinogenic potency of PAHs (*6*) and has been useful in detecting the carcinogenic activity of chemicals possibly related to ethyl carbamate in terms of their mechanism of action, such as vinyl carbamate and acrylamide (*3*).

The present results suggest that the development of papillomas was predictive of the later development of carcinomas. This finding is in essential agreement with the data reviewed by Burns et al. (*7*), which indicated a 5 to 7% conversion of papillomas to carcinomas. To the extent that it could be ascertained, this relationship did not appear to depend on the individual chemical. There was as much scatter in the relationship when a single chemical was involved (B[a]P) in multiple tests (Fig. 2*B*) as when a variety of chemicals were involved in the tests (Fig. 2*C*). It should be noted, however, that no simple linear relationship exists between the development of papillomas at 24 weeks and carcinoma incidence at 52 weeks. It was clear that circumstances involving low papilloma yields (including animals that received only TPA treatments) had a higher likelihood of yielding carcinomas relative to the papilloma yield than when high papilloma yields were involved. One must be cautious in interpreting these data because the lethality of malignant tumors biases the results at high papilloma yields. Nevertheless this curvilinear relationship appears to be present in papilloma yields where survival was not limiting (i.e., < 4 papillomas/animal).

The research performed in this paper has been peer and administratively reviewed by the U.S. Environmental Protection Agency and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

REFERENCES

1. Bull, R. J., and Pereira, M. A. Development of a short-term testing matrix for estimating carcinogenic risk. J. Amer. Coll. Toxicol. 1: 1–15 (1982).

2. Bull, R. J., Meier, J. R., Robinson, M., Ringhand, H. P. Laurie, R. D., and Stober, J. Evaluation of mutagenic and carcinogenic properties of brominated and chlorinated acetonitriles: by-products of chlorination. Fundam. Appl. Toxicol. 5: 1065–1074 (1985).
3. Bull, R. J., Robinson, M., Laurie, R. D., Stoner, G. D., Greisiger, E., Meier, J. R., and Stober, J. Carcinogenic effects of acrylamide in SENCAR and A/J mice. Cancer Res. 44: 107–111 (1984).
4. Weisburger, J. H., and Williams, G. M. Decision point approach to carcinogen testing. In: Structural Correlates of Carcinogenesis and Mutagenesis. HEW Publication No. (FDA) 78-1046, Rockville, MD, 1977, pp. 45–52.
5. Periera, M. A. Mouse skin bioassay for chemical carcinogens. J. Amer. Coll. Toxicol. 1: 47–82 (1982).
6. Slaga, T. J., Fischer, S. M., Triplett, L. L., and Nesnow, S. Comparison of complete carcinogenesis and tumor initiation and promotion in mouse skin: the induction of papillomas by tumor initiation-promotion a reliable short term assay. J. Amer. Coll. Toxicol. 1: 83–99 (1982).
7. Burns, F. J., Albert, R. E., and Altshuler, B. Cancer progression in mouse skin. In: Mechanisms of Tumor Promotion. Volume II. Tumor Promotion and Skin Carcinogenesis (T. J. Slaga, Ed.), CRC Press Inc., Boca Raton, FL, 1984, pp. 17–39.